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Twentieth day of November 2003

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S&F Ref: 609332

ORIGINAL

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION FOR THE INVENTION ENTITLED:

Induction of the Mitochondrial Permeability Transition

Name and Address of Applicant:

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This invention is best described in the following statement:

Induction of the Mitochondrial Permeability Transition

Technical Field

The present invention relates to modification of mitochondrial membrane permeability in cells and in particular, the identification and use of compounds which selectively induce the MPT in proliferating cells.

Background of the Invention

Mitochondria provide ATP to support normal cell function and their perturbation leads to apoptotic and necrotic cell death (Crompton, 1999). An important factor in apoptosis and necrosis is the mitochondrial permeability transition (MPT), which occurs as a result of calcium overload. The cause of the MPT is the opening of a non-specific pore in the inner mitochondrial membrane, known as the mitochondrial permeability transition pore (MPTP) (Crompton et al., 1987). Oxidative stress, adenine nucleotide depletion and elevated inorganic phosphate greatly increase the sensitivity of the pore to calcium concentration. Opening of the MPTP is accompanied by equilibration of all small solutes (< 1.5 kD) across the inner mitochondrial membrane. The resultant high protein concentration in the matrix exerts a colloidal osmotic pressure that is responsible for extensive swelling of mitochondria, and ultimately, apoptosis or necrosis.

Adenine nucleotide translocator (ANT) is a 30 kD protein that spans the inner mitochondrial membrane and is central to the MPTP (Crompton et al., 1988).

There is a need to selectively induce the MPT, particularly in proliferating cells, as a means of inducing apoptosis.

The present invention relates to a process for identifying compounds which bind to ANT in mitochondria and selectively induce the MPT in proliferating cells relative to non-proliferating or growth quiescent cells.

Summary of the Invention

According to a first embodiment of the invention there is provided a process for identifying a compound which induces the mitochondrial permeability transition (MPT) in proliferating cells, wherein the process comprises contacting a cell or cell extract with a compound, determining whether the compound binds to adenine nucleotide translocator (ANT), and determining whether the compound selectively induces the MPT in proliferating cells.

According to a second embodiment of the invention there is provided a process for screening a plurality of compounds to identify a compound which induces MPT in proliferating cells, wherein the process comprises contacting a cell or a cell extract with the plurality of compounds,

determining whether any of the compounds bind to ANT, and if so, separately determining for each of the plurality of compounds whether the compound selectively induces the MPT in proliferating cells.

Typically, with regard to the first and second embodiments of the invention, selectivity for proliferating cells can be determined by comparing the effect of compounds identified as binding to ANT in accordance with the first or second embodiment of the invention, on the MPT in proliferating cells with the effect on the MPT in non-proliferating or growth quiescent cells.

According to a third embodiment of the invention there is provided a process for identifying a compound which induces apoptosis in proliferating cells, the process comprising contacting a cell or cell extract with a candidate compound, determining whether there is an increase in cellular superoxide anion (O_2^-) concentration, and determining whether the compound selectively induces apoptosis in proliferating cells.

According to a fourth embodiment of the invention there is provided a process for identifying a compound which is an inhibitor of angiogenesis, the process comprising contacting a cell or cell extract with a candidate compound, determining whether there is an increase in cellular superoxide anion (O_2^-) concentration, and determining whether the compound is an inhibitor of angiogenesis.

According to a fifth embodiment of the invention there is provided a method of inducing MPT in a vertebrate, wherein the method comprises administering to the vertebrate a therapeutically effective amount of at least one compound detected in accordance with the process of any one of the first to third embodiments of the invention, or a therapeutically effective amount of a pharmaceutical composition comprising at least one of the compounds together with a pharmaceutically acceptable carrier, adjuvant and/or diluent.

According to a sixth embodiment of the invention there is provided a method of inducing apoptosis in proliferating mammalian cells, comprising administering to the mammal an apoptosis-inducing amount of a compound identified in accordance with the any one of the first to third embodiments of the invention, or a therapeutically effective amount of a pharmaceutical composition comprising at least one of the compounds together with a pharmaceutically acceptable carrier, adjuvant and/or diluent.

According to a seventh embodiment of the invention there is provided the use of a compound identified in accordance with any one of the first to third embodiments of the invention for the manufacture of a medicament for inducing apoptosis.

According to an eighth embodiment of the invention there is provided a method of inhibiting angiogenesis in a mammal, comprising administering to the mammal an angiogenesis-inhibiting amount of a compound identified in accordance with the fourth embodiment of the invention, or a

therapeutically effective amount of a pharmaceutical composition comprising at least one of the compounds together with a pharmaceutically acceptable carrier, adjuvant and/or diluent.

According to a ninth embodiment of the invention there is provided the use of a compound identified in accordance with the fourth embodiment of the invention for the manufacture of a medicament for inhibiting angiogenesis.

With reference to any one of the first to ninth embodiments of the invention, typically the compound is a dithiol reactive compound.

Still typically, the compound has an arsenoxide (or arsenoxide equivalent) moiety.

Still typically, the arsenoxide (or arsenoxide equivalent) compound is of the formula (I):

$$A\text{-}(L\text{-}Y)_p \qquad (I)$$

wherein

A comprises at least one pendant group;

L comprises any suitable linker and/or spacer group;

Y comprises at least one arsenoxide or arsenoxide equivalent;

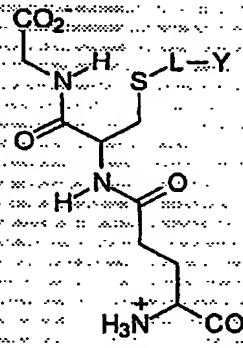
p is an integer from 1 to 10.

Typically, the compound of formula (I) has more than 6 carbon atoms.

With reference to the compound of formula (I), typically, A is selected from the group consisting of natural, unnatural and synthetic amino acids, hydrophilic amines, peptides, polypeptides, oligosaccharides, and thiol containing proteins, or a combination thereof. More typically, A is selected from the group consisting of glutathione, glucosamine, cysteinylglycine, cysteic acid, aspartic acid, glutamic acid, lysine, and arginine, and wherein the sulfur atom of each sulfur containing compound may be optionally oxidised to form a sulfoxide or sulfone.

Amino acid side chains are known to those of skill in the art and are listed, for example, in standard reference texts such as King and Stansfield, "A Dictionary of Genetics", 4th Edition, Oxford University Press, 1990, the contents of which are incorporated herein by reference.

Even more typically, A is glutathione and in one form the compound is represented by Formula (II):



(II)

wherein L comprises any suitable linker and/or spacer group, and Y comprises an arsenoxide or an arsenoxide equivalent.

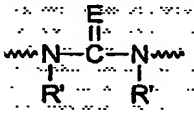
Typically, p is an integer from 1 to 5. Yet still more typically, p is 1.

Typically, L corresponds to $(XBX')_nB'$. Typically, n is an integer from 0 to 20, more typically 0 to 15, even more typically 0 to 10, still more typically 0 to 5.

Still typically, X is selected from the group consisting of –NR, -S(O)-, -S(O)O-, $-S(O)_2$-, $-S(O)_2O$-, -C(O)-, -C(S)-, -C(O)O-, C(S)O-, -C(S)S-, $-P(O)(R_1)$-, and $-P(O)(R_1)O$-, or is absent;

B is selected from the group consisting of C_1-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, C_3-C_{10} cycloalkylene, C_5-C_{10} cycloalkenylene, C_3-C_{10} heterocycloalkylene, C_5-C_{10} heterocycloalkenylene, C_6-C_{12} arylene, heteroarylene and C_2-C_{10} acyl;

X' is selected from the group consisting of -NR-, –O-, -S-, -Se-, -S-S-, S(O)-, -OS(O)-, -OS(O)O-, $-OS(O)_2$, $-OS(O)_2O$-, $-S(O)_2$-, $-S(O)_2O$-, $-OP(O)(R_1)$-, $-OP(O)(R_1)O$-, $-OP(O)(R_1)OP(O)(R_1)O$-, -C(O)-, -C(S)-, -C(O)O-, C(S)O-, -C(S)S-, $-P(O)(R_1)$-, $-P(O)(R_1)O$-, and



or is absent; wherein E is O, S, Se, NR or $N(R)_2^+$;

n is 0, 1 or 2; and

B' is selected from the group consisting of C_1-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, C_3-C_{10} cycloalkylene, C_5-C_{10} cycloalkenylene, C_3-C_{10} heterocycloalkylene, C_5-C_{10} heterocycloalkenylene, C_6-C_{12} arylene, and heteroarylene or is absent; and wherein

each R is independently selected from the group consisting of hydrogen, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_3-C_{10} heterocycloalkyl, C_5-C_{10} heterocycloalkenyl, C_6-C_{12} aryl, heteroaryl, OR_2 and C_2-C_{10} acyl;

R' is the same as R or two R' may be taken together with the nitrogen atoms to which they are attached to form a 5 or 6-membered saturated or unsaturated heterocyclic ring;

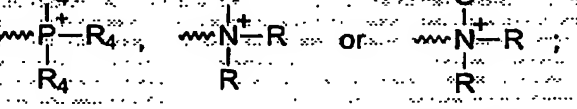
each R_1 is independently selected from the group consisting of hydrogen, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_3-C_{10} heterocycloalkyl, C_5-C_{10} heterocycloalkenyl, C_6-C_{12} aryl, heteroaryl, halo, OR_2 and $N(R)_2$;

each R_2 is independently selected from the group consisting of hydrogen, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_3-C_{10} heterocycloalkyl, C_5-C_{10} heterocycloalkenyl, C_6-C_{12} aryl, heteroaryl and -$C(O)R_5$;

each R_5 is independently selected from the group consisting of hydrogen, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_3-C_{10} heterocycloalkyl, C_5-C_{10} heterocycloalkenyl, C_6-C_{12} aryl, heteroaryl, C_1-C_{10} alkoxy, C_3-C_{10} alkenyloxy, C_3-C_{10} alkynyloxy, C_3-C_{10} cycloalkyloxy, C_5-C_{10} cycloalkenyloxy, C_3-C_{10} heterocycloalkyloxy, C_5-C_{10} heterocycloalkenyloxy, C_6-C_{12} aryloxy, heteroaryloxy, C_1-C_{10} alkylthio, C_3-C_{10} alkenylthio, C_3-C_{10} alkynylthio, C_3-C_{10} cycloalkylthio, C_5-C_{10} cycloalkenylthio, C_3-C_{10} heterocycloalkylthio, C_5-C_{10} heterocycloalkenylthio, C_6-C_{12} arylthio, heteroarylthio, OH, SH and $N(R)_2$;

wherein for each instance that B and/or B' is arylene, the substituents directly attached to the respective arylene rings (including arsenoxide or arsenoxide equivalent) may be in a para-, meta- or ortho- relationship; and

wherein each alkylene, alkenylene, alkynylene, cycloalkylene, cycloalkenylene, heterocycloalkylene, heterocycloalkenylene, arylene, heteroarylene and acyl may be independently substituted with hydrogen, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_3-C_{10} heterocycloalkyl, C_5-C_{10} heterocycloalkenyl, C_6-C_{12} aryl, heteroaryl, cyano, cyanate, isocyanate, OR_{2a}, SR_6, nitro, arsenoxide, -$S(O)R_3$, -$OS(O)R_3$, -$S(O)_2R_3$, -$OS(O)_2R_3$, -$P(O)R_4R_4$, -$OP(O)R_4R_4$, -$N(R")_2$, -$NRC(O)(CH_2)_mQ$, -$C(O)R_5$;



wherein R, R_1 and R_5 are as defined above; and

R_{2a} is selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, -$S(O)R_3$, -$S(O)_2R_3$, -$P(O)(R_4)_2$, $N(R)_2$ and -$C(O)R_5$;

each R_3 is independently selected from the group consisting of hydrogen, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_3-C_{10} heterocycloalkyl, C_5-C_{10} heterocycloalkenyl, C_6-C_{12} aryl, heteroaryl, C_1-C_{10} alkoxy, C_3-C_{10} alkenyloxy, C_3-C_{10} alkynyloxy, C_3-C_{10} cycloalkyloxy, C_5-C_{10} cycloalkenyloxy, C_3-C_{10} heterocycloalkyloxy, C_5-C_{10} heterocycloalkenyloxy, C_6-C_{12} aryloxy, heteroaryloxy, C_1-C_{10} alkylthio, C_3-C_{10} alkenylthio, C_3-C_{10}

alkynylthio, C_3-C_{10} cycloalkylthio, C_5-C_{10} cycloalkenylthio, C_3-C_{10} heterocycloalkylthio, C_5-C_{10} heterocycloalkenylthio, C_6-C_{12} arylthio, heteroarylthio and $N(R)_2$;

each R_4 is independently selected from the group consisting of hydrogen, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_3-C_{10} heterocycloalkyl, C_5-C_{10} heterocycloalkenyl, C_6-C_{12} aryl, heteroaryl, C_1-C_{10} alkoxy, C_3-C_{10} alkenyloxy, C_3-C_{10} alkynyloxy, C_3-C_{10} cycloalkyloxy, C_5-C_{10} cycloalkenyloxy, C_3-C_{10} heterocycloalkyloxy, C_5-C_{10} heterocycloalkenyloxy, C_6-C_{12} aryloxy, heteroaryloxy, C_1-C_{10} alkylthio, C_3-C_{10} alkenylthio, C_3-C_{10} alkynylthio, C_3-C_{10} cycloalkylthio, C_5-C_{10} cycloalkenylthio, C_3-C_{10} heterocycloalkylthio, C_5-C_{10} heterocycloalkenylthio, C_6-C_{12} arylthio, heteroarylthio, halo and $N(R)_2$;

R_6 is selected from the group consisting of C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_3-C_{10} heterocycloalkyl, C_5-C_{10} heterocycloalkenyl, C_6-C_{12} aryl, heteroaryl, C_1-C_{10} alkylthio, C_3-C_{10} alkenylthio, C_3-C_{10} alkynylthio, C_3-C_{10} cycloalkylthio, C_5-C_{10} cycloalkenylthio, C_3-C_{10} heterocycloalkylthio, C_5-C_{10} heterocycloalkenylthio, C_6-C_{12} arylthio, heteroarylthio, $-S(O)R_3$, $-S(O)_2R_3$ and $-C(O)R_5$;

R" is the same as R or two R" taken together with the N atom to which they are attached may form a saturated, unsaturated or aromatic heterocyclic ring system;

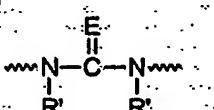
Q is selected from halogen and $-OS(O)_2Q_1$; wherein Q_1 is selected from C_1-C_4 alkyl, C_1-C_4 perfluoroalkyl, phenyl, *p*-methylphenyl; and

m is 1 to 5.

More typically, X is selected from the group consisting of -C(O)-, -C(S)-, -C(O)O-, C(S)O-, and -C(S)S-, or is absent;

B is selected from the group consisting of C_1-C_5 alkylene, C_2-C_5 alkenylene, C_2-C_5 alkynylene, C_3-C_{10} cycloalkylene, C_5-C_{10} cycloalkenylene, C_6-C_{12} arylene and C_2-C_5 acyl;

X' is selected from the group consisting of –O-, -S-, -NR-, -S-S-, -S(O)-, $-S(O)_2$-, $-P(O)(R_1)$-, $-OP(O)(R_1)$-, $OP(O)(R_1)O$-, $-OP(O)(R_1)OP(O)(R_1)O$-, -C(O)-, -C(S)-, -C(O)O-, C(S)O-, -C(S)S-, -Se-



, or is absent; wherein E is O, S or $N(R)_2^+$;

n is 0, 1 or 2; and

B' is C_1-C_5 selected from the group consisting of alkylene, C_2-C_5 alkenylene, C_2-C_5 alkynylene, C_3-C_{10} cycloalkylene, C_5-C_{10} cycloalkenylene, and C_6-C_{12} arylene, or is absent; and wherein

each R is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, OR_2 and C_2-C_{10} acyl;

R' is the same as R;

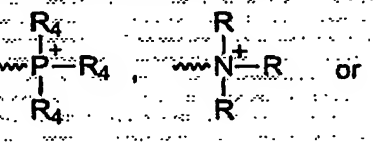
each R_1 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, halo, OR_2 and $N(R)_2$;

each R_2 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, and $-C(O)R_5$;

each R_5 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, C_3-C_5 alkenyloxy, C_3-C_5 alkynyloxy, C_3-C_{10} cycloalkyloxy, C_5-C_{10} cycloalkenyloxy, C_6-C_{12} aryloxy, C_1-C_5 alkylthio, C_3-C_5 alkenylthio, C_3-C_5 alkynylthio, C_3-C_{10} cycloalkylthio, C_5-C_{10} cycloalkenylthio, C_6-C_{12} arylthio, OH, SH, and $N(R)_2$;

wherein for each instance that B and/or B'' is arylene, the substituents directly attached to the respective arylene rings (including arsenoxide or arsenoxide equivalent), may be in a para-, meta- or ortho- relationship, and

wherein each alkylene, alkenylene, alkynylene, cycloalkylene, cycloalkenylene, arylene, and acyl may be independently substituted with hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, cyano, halo, cyanate, isocyanate, OR_{2a}, SR_6, nitro, arsenoxide, $-S(O)R_3$, $-OS(O)R_3$, $-S(O)_2R_3$, $-OS(O)_2R_3$, $-P(O)R_4R_4$, $-OP(O)R_4R_4$, $-N(R'')_2$, $NRC(O)(CH_2)_mQ$, $-C(O)R_5$;



wherein R, R_1 and R_5 are as defined above; and

R_{2a} is selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, $-S(O)R_3$, $-S(O)_2R_3$, $-P(O)(R_4)_2$, $N(R)_2$ and $-C(O)R_5$;

each R_3 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, C_3-C_5 alkenyloxy, C_3-C_5 alkynyloxy, C_3-C_{10} cycloalkyloxy, C_5-C_{10} cycloalkenyloxy, C_6-C_{12} aryloxy, C_1-C_5 alkylthio, C_3-C_5 alkenylthio, C_3-C_5 alkynylthio, C_3-C_{10} cycloalkylthio, C_5-C_{10} cycloalkenylthio, C_6-C_{12} arylthio and $N(R)_2$;

each R_4 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, C_3-C_5

alkenyloxy, C_3-C_5 alkynyloxy, C_3-C_{10} cycloalkyloxy, C_5-C_{10} cycloalkenyloxy, C_6-C_{12} aryloxy, C_1-C_5 alkylthio, C_3-C_5 alkenylthio, C_3-C_5 alkynylthio, C_3-C_5 cycloalkylthio, C_5-C_5 cycloalkenylthio, C_6-C_{12} arylthio, halo and $N(R)_2$;

R_8 is independently selected from the group consisting of C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, C_1-C_5 alkylthio, C_3-C_5 alkenylthio, C_3-C_5 alkynylthio, C_3-C_{10} cycloalkylthio, C_5-C_{10} cycloalkenylthio, C_6-C_{12} arylthio, $-S(O)R_3$, $-S(O)_2R_3$ and $-C(O)R_5$,

R'' is the same as R;

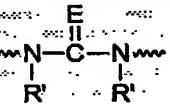
Q is selected from the group consisting of halogen and $-OS(O)_2Q_1$, wherein Q_1 is selected from C_1-C_4 alkyl, C_1-C_4 perfluoroalkyl, phenyl, *p*-methylphenyl; and

m is 1 to 5.

Even more typically, X is absent;

B is selected from the group consisting of C_1-C_5 alkylene, C_6-C_{12} arylene and C_2-C_5 acyl;

X' is selected from the group consisting of –O-, -S-, -NR-, -S-S-, -S(O)-, $-S(O)_2$-, $-P(O)(R_1)$-, -C(O)-, -C(S)-, -C(O)O-, C(S)O-, -Se-, and



, or absent; wherein E is O, S or $N(R)_2^+$;

n is 0, 1 or 2; and

B' is C_1-C_5 alkylene, C_6-C_{12} arylene or is absent; and wherein

each R is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_3-C_{10} cycloalkyl, C_6-C_{12} aryl, OR_2 and C_2-C_5 acyl;

R' is the same as R;

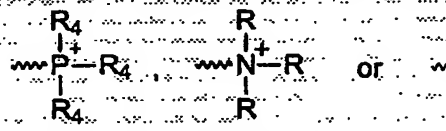
each R_1 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_3-C_{10} cycloalkyl, C_6-C_{12} aryl, halo, OR_2 and $N(R)_2$;

each R_2 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_3-C_{10} cycloalkyl, C_6-C_{12} aryl and $-C(O)R_5$;

each R_5 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, C_3-C_5 alkenyloxy, C_3-C_{10} cycloalkyloxy, C_5-C_{10} cycloalkenyloxy, C_6-C_{12} aryloxy, C_1-C_5 alkylthio, C_3-C_5 alkenylthio, C_3-C_{10} cycloalkylthio, C_5-C_{10} cycloalkenylthio, C_6-C_{12} arylthio, OH, SH and $N(R)_2$;

wherein for each instance that B and/or B' is arylene, the substituents directly attached to the respective arylene rings (including arsenoxide or arsenoxide equivalent) may be in a para-, meta- or ortho- relationship, and

wherein each alkylene, alkenylene, alkynylene, cycloalkylene, cycloalkenylene, arylene, and acyl may be independently substituted with hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, halo, cyano, cyanate, isocyanate, OR_{2a}, SR_6, nitro, arsenoxide, $-S(O)R_3$, $-OS(O)R_3$, $-S(O)_2R_3$, $-OS(O)_2R_3$, $-P(O)R_4R_4$, $-OP(O)R_4R_4$, $-N(R')_2$, $-NRC(O)(CH_2)_mQ$, $-C(O)R_5$,



wherein R, R_1 and R_5 are as defined above; and

R_{2a} is selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_3-C_{10} cycloalkyl, C_6-C_{12} aryl, $-S(O)R_3$, $-S(O)_2R_3$, $-P(O)(R_4)_2$ and $-C(O)R_5$;

each R_3 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_3-C_{10} cycloalkyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, C_3-C_{10} cycloalkyloxy, C_6-C_{12} aryloxy, C_1-C_5 alkylthio, C_3-C_{10} cycloalkylthio, C_6-C_{12} arylthio and $N(R)_2$;

each R_4 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_3-C_{10} cycloalkyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, C_3-C_{10} cycloalkyloxy, C_6-C_{12} aryloxy, halo and $N(R)_2$;

R_6 is selected from the group consisting of C_1-C_5 alkyl, C_3-C_{10} cycloalkyl, C_6-C_{12} aryl, C_1-C_5 alkylthio, C_3-C_{10} cycloalkylthio, C_6-C_{12} arylthio, $-S(O)R_3$, $-S(O)_2R_3$ and $-C(O)R_5$;

R' is the same as R;

Q is selected from halogen and $-OS(O)_2Q_1$; wherein Q_1 is selected from C_1-C_4 alkyl, C_1-C_4 perfluoroalkyl, phenyl, *p*-methylphenyl; and

m is 1 to 5;

Still more typically, X is absent;

B is selected from the group consisting of C_1-C_5 alkylene, C_6-C_{12} arylene and C_2-C_5 acyl;

X' is selected from the group consisting of –O-, -S-, -NR-, -C(O)-, and -C(O)O-, or is absent;

n is 1; and

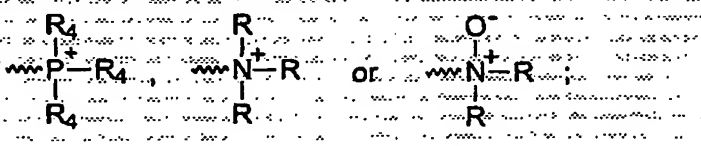
B' is C_1-C_5 alkylene, C_6-C_{12} arylene or is absent; and

R is selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_6-C_{12} aryl and C_2-C_5 acyl;

wherein for each instance that B and/or B' is arylene, the substituents directly attached to the respective arylene rings (including arsenoxide or arsenoxide equivalent), may be in a para-, meta- or ortho- relationship, and

wherein each alkylene, arylene, and acyl may be independently substituted with hydrogen, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, C_3-C_{10} cycloalkyl, C_5-C_{10} cycloalkenyl, C_6-C_{12} aryl, halo,

cyano, cyanate, isocyanate, OR_{2a}, SR_6, nitro, arsenoxide, $-S(O)R_3$, $-S(O)_2R_3$, $-P(O)R_4R_4$, $-N(R'')_2$, $-NRC(O)(CH_2)_mQ$, $-C(O)R_5$,



wherein each R is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_6-C_{12} aryl and C_2-C_5 acyl;

R_{2a} is selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_6-C_{12} aryl, $-S(O)R_3$, $-S(O)_2R_3$, $-P(O)(R_4)_2$ and $-C(O)R_5$;

each R_3 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, C_6-C_{12} aryloxy, C_1-C_5 alkylthio, and C_6-C_{12} arylthio;

each R_4 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, C_6-C_{12} aryloxy, C_1-C_5 alkylthio, C_6-C_{12} arylthio, halo and $N(R)_2$;

each R_5 is independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, C_6-C_{12} aryloxy, C_1-C_5 alkylthio, C_6-C_{12} arylthio, OH, SH and $N(R)_2$;

R_6 is selected from the group consisting of C_1-C_5 alkyl, C_6-C_{12} aryl, C_1-C_5 alkylthio, C_6-C_{12} arylthio, $-S(O)R_3$, $-S(O)_2R_3$ and $-C(O)R_5$

R'' is the same as R above;

Q is selected from halogen and $-OS(O)_2Q_1$; wherein Q_1 is selected from C_1-C_4 alkyl, C_1-C_4 perfluoroalkyl, phenyl, *p*-methylphenyl; and

m is 1 to 5.

Yet still more typically, X is absent;

B is C_2-C_5 acyl;

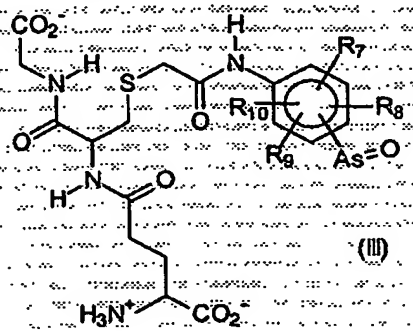
X' is NR;

n is 1;

B' is phenylene; and

R is H;

wherein the substituents directly attached to the phenylene ring may be in a para-, meta- or ortho- relationship, as exemplified by Formula (III):



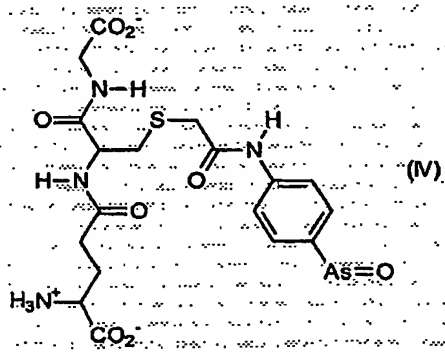
(III)

wherein R_7 to R_{10} are independently selected from the group consisting of hydrogen, C_1-C_5 alkyl, C_6-C_{12} aryl, halogen, hydroxy, amino, nitro, carboxy, C_1-C_5 alkoxy, $-OS(O)_2R_3$ and $-NHC(O)CH_2Q$ wherein Q is halogen, $-OS(O)_2CH_3$, $-OS(O)_2C_6H_5$ and $-OS(O)_2$-*p* tolyl; and wherein, when any one of R_7 to R_{10} is C_1-C_5 alkyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, $-OS(O)_2R_3$ it is capable of forming a fused ring with the phenylene; and further wherein, at least one of R_7 to R_{10} is C_1-C_5 alkyl, C_6-C_{12} aryl, C_1-C_5 alkoxy, or $-OS(O)_2R_3$, in combination with at least any one other of R_7 to R_{10}, is capable of forming a fused ring with the phenylene.

More typically, R_7 to R_{10} are independently selected from the group consisting of hydrogen, halogen, hydroxy, amino, nitro, cyano, carboxy, C_1-C_5 alkoxy, methyl, ethyl, isopropyl, tert-butyl, phenyl and $-NHC(O)CH_2Q$ wherein Q is halogen, $-OS(O)_2CH_3$, $-OS(O)_2C_6H_5$ and $-OS(O)_2$-*p* tolyl.

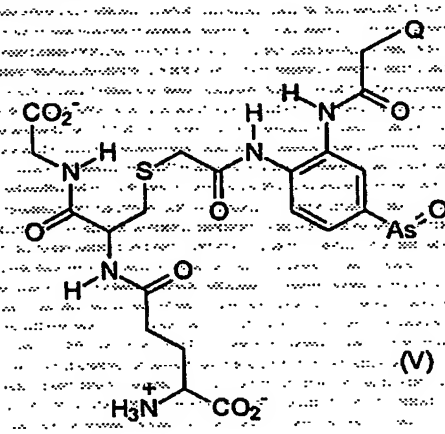
Further, when B' is arylene, the substituents attached to the arylene ring are typically in an ortho-, meta- or para- relationship to the –As=O. More typically the substituents are in a meta- or para- relationship to the –As=O group.

More typically an arsenoxide compound identified according to the present invention is 4-(*N*-(S-glutathionylacetyl)amino)-phenylarsenoxide, which can be abbreviated to GSAO, according to Formula IV:



(IV)

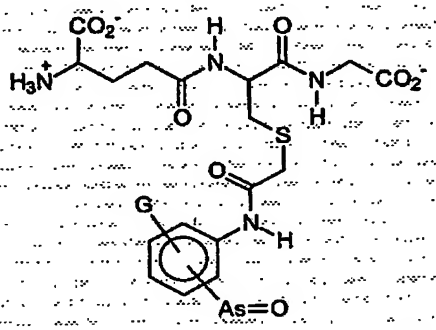
Other arsenoxide compounds which interact with ANT include compounds according to Formula (V):



(V)

wherein Q is any halogen.

Another typical form of an arsenoxide compound which interacts with ANT is a compound according to Formula (VI):



(VI)

wherein G is selected from the group consisting of hydrogen, halogen, hydroxy, amino, nitro, cyano, carboxy, C_1-C_5 alkoxy, C_1-C_5 alkyl and C_6-C_{12} aryl and -$NHC(O)CH_2Q$, wherein Q is halogen, $-OS(O)_2CH_3$, $-OS(O)_2C_6H_5$ or $-OS(O)_2$-*p* tolyl.

Typically, G is selected from the group consisting of hydrogen, halogen, hydroxy, amino, nitro, carboxy, C_1-C_5 alkoxy, methyl, ethyl, isopropyl, tert-butyl, phenyl, and -$NHC(O)CH_2Q$, wherein Q is the group consisting of halogen, $-OS(O)_2CH_3$, $-OS(O)_2C_6H_5$ and $-OS(O)_2$-*p* tolyl.

More typically, in a compound of Formula VI, G is hydroxy, fluorine, amino, or nitro.

Typically, group G is in an ortho-, meta- or para- relationship to the arsenoxide group, more typically an ortho- or para- relationship.

Typically the activity of the arsenic atom may be modified by the group G, when G and the arsenic atom are in an ortho or para relationship to one another. For example, when G is an electron donating group such as OH (ionised to O^- at physiological pH), the arsenic atom should be deactivated towards dithiols and so become more selective, only reacting with very reactive dithiols. Alternatively, when G is an electron withdrawing group, such as NO_2, electron density would be

drawn away from the arsenic atom, making it more reactive to all dithiols. Selective inhibition of some redox proteins and not others may be achieved by manipulation of G.

Typically, in the arsenoxide compounds capable of interacting with ANT, the arsenoxide group (-As=O) can be replaced by an arsenoxide equivalent.

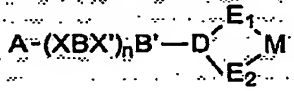
An arsenoxide equivalent is any dithiol reactive species that shows essentially the same affinity towards dithiols as –As=O. Typically, arsenoxide equivalent includes dithiol reactive entities, such as As, Ge, Sn and Sb species. More typically an arsenoxide equivalent can be represented by -D(Z_1)(Z_2). Arsenoxide equivalents are expected to exhibit identical or substantially identical activity to that of the corresponding arsenoxide.

Typically, for arsenoxide equivalents of the form -D(Z_1)(Z_2), D will be, for example, As, RSn, Sb, or RGe, and Z_1 and Z_2 will be labile groups (i.e. groups easily displaced under physiological conditions). Z_1 and Z_2 may be identical or different, and may either be connected or independent from each other (bound only to the arsenic atom).

Suitable arsenoxide equivalents include the following:

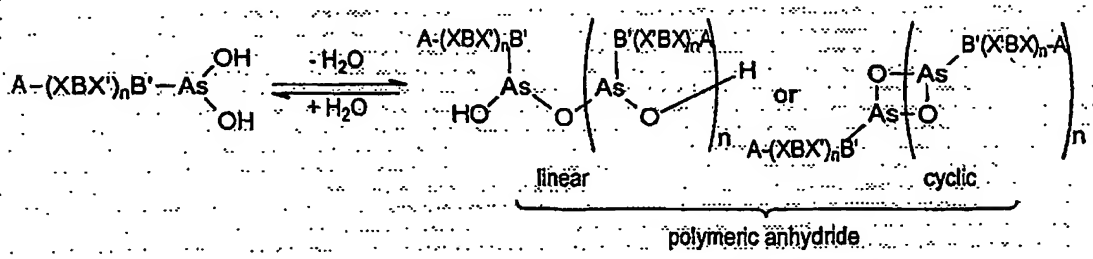
-D(Z_1)(Z_2),

wherein Z_1 and Z_2 are selected from the group consisting of OH, C_1-C_{10} alkoxy, C_6-C_{10} aryloxy, C_1-C_{10} alkylthio, C_6-C_{10} arylthio, C_1-C_{10} alkylseleno, C_6-C_{10} arylseleno, F, Cl, Br and I;



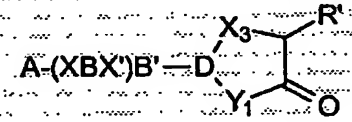
wherein E_1 = E_2 = O, E_1 =O and E_2 = S or E_1 = E_2 = S; M is R''' and R'''' are independently selected from the group consisting of hydrogen, C_1-C_{10} alkyl, C_6-C_{12} aryl, halogen, C_1-C_{10} alkoxy, C_6-C_{10} aryloxy, hydroxy and carboxy; and n = 1 to 10.

For arsenoxide equivalents of the form D(Z_1)(Z_2), when D is As and Z_1 and Z_2 are OH, the arsenoxide equivalent may be in equilibrium with polymeric species, as depicted below.

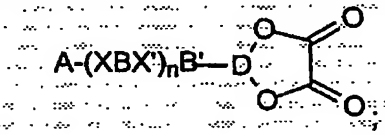


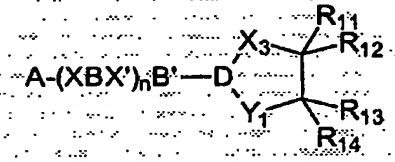
In respect of the equilibrium depicted above, arsenic is one of many elements whose hydroxy species exist in equilibrium with the corresponding polymeric anhydrides (Doak & Freedman, 1970). Therefore, arsenoxide compounds may actually exist as low or medium molecular weight polymers (eg n = 3 to 6). However, the dehydration reaction is reversible, and therefore soluble

polymeric anhydrides are expected to behave as arsenoxide equivalents, that is, they are expected to bind to closely spaced dithiols in substantially the same way as the monomeric $-As(OH)_2$ species.

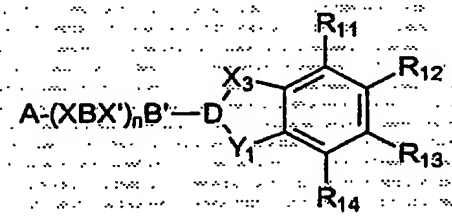


wherein X_3=NH, Y_1=O; X_3=Y_1=O or X_3=S, Y_1=O, and R' is selected from the group consisting of hydrogen, C_1-C_{10} alkyl, C_6-C_{12} aryl, and carboxy, or is one of the twenty amino acid side chains;





wherein X_3=Y_1=O; X_3=NH, Y_1=O; X_3=S, Y_1=O; X_3=Y_1=NH; or X_3=S, Y_1=NH or X_3=S, Y_1=NH and R_{11} to R_{14} are selected from the group consisting of hydrogen, C_1-C_{10} alkyl, C_6-C_{12} aryl, and CO_2H;



wherein X_3=Y_1=O, or X_3=NH, Y_1=O; and R_{11} to R_{14} are selected from the group consisting of hydrogen, C_1-C_{10} alkyl, C_6-C_{12} aryl, halogen, C_1-C_{10} alkoxy, and CO_2H.

Typically, (XBX')B' is as defined above.

Abbreviations and Definitions

In the context of this specification, the abbreviation MPT stands for Mitochondrial Permeability Transition.

In the context of this specification, the abbreviation ANT stands for adenine nucleotide translocator.

In the context of this specification EGTA is ethylene glycol-bis(beta-aminoethyl ether)-N,N,N'N'-tetraacetic acid.

In the context of this specification, PAO stands for phenyl arsenoxide.

In the context of this specification, the term "comprising" means "including principally, but not necessarily solely". Furthermore, variations of the word "comprising", such as "comprise" and "comprises", have correspondingly varied meanings.

In the context of this specification, the term "arsenoxide" refers to the group –As=O.

In the context of this specification, the groups written –As=O and –As(OH)$_2$ are to be considered synonymous.

In the context of this specification, the term "arsenoxide equivalent" refers to any dithiol reactive species that shows essentially the same affinity towards dithiols as –As=O or As(OH)$_2$, and the term includes, for example, groups comprising a transition element, and any trivalent arsenical that is either hydrolysed to –As=O or –As(OH)$_2$ when dissolved in an aqueous medium (such as cell culture buffers and the fluids contained in the organism being treated).

The term "arsenical" as used herein, includes any compound that contains arsenic.

The term "acyl" as used herein, includes monovalent and divalent alkyl, alkenyl, alkynyl, cycloalkyl and cycloalkenyl moieties possessing a terminal carbonyl substituent wherein attachment may occur at the hydrocarbon moiety, the carbonyl moiety or both.

The term "alkyl" as used herein, includes within its meaning monovalent, saturated, straight and branched chain hydrocarbon radicals.

The term "alkenyl" as used herein, includes within its meaning, monovalent, straight and branched chain hydrocarbon radicals having at least one double bond.

The term "alkynyl" as used herein, includes within its meaning, monovalent, straight and branched chain hydrocarbon radicals having at least one triple bond.

The term "alkylene" as used herein, includes within its meaning divalent, saturated, straight chain hydrocarbon radicals.

The term "alkenylene" as used herein, includes within its meaning, divalent, straight chain hydrocarbon radicals having at least one double bond.

The term "alkynylene" as used herein, includes within its meaning, divalent, straight chain hydrocarbon radicals having at least one triple bond.

The term "aryl" as used herein, includes within its meaning monovalent, single, polynuclear, conjugated and fused aromatic hydrocarbon radicals.

The term "arylene" as used herein, includes within its meaning divalent, single, polynuclear, conjugated and fused aromatic hydrocarbon radicals.

The term "closely spaced dithiol" as used herein, includes within its meaning thiols that are chemically vicinal, as well as thiols brought into spacial apposition by virtue of molecular conformation.

The term "cycloalkyl" as used herein, includes within its meaning monovalent, saturated, monocyclic, bicyclic, polycyclic or fused polycyclic hydrocarbon radicals.

The term "cycloalkylene" as used herein, includes within its meaning divalent, saturated, monocyclic, bicyclic, polycyclic or fused polycyclic hydrocarbon radicals.

The term "cycloalkenyl" as used herein, includes within its meaning monovalent, saturated, monocyclic, bicyclic, polycyclic or fused polycyclic hydrocarbon radicals having at least one double bond.

The term "cycloalkenylene" as used herein, includes within its meaning divalent, saturated, monocyclic, bicyclic, polycyclic or fused polycyclic hydrocarbon radicals having at least one double bond.

The term "halo" as used herein, includes fluoro, chloro, bromo and iodo.

The term "heteroaryl" as used herein, includes within its meaning monovalent, single, polynuclear, conjugated and fused aromatic radicals having 1 to 12 atoms wherein 1 to 6 atoms are heteroatoms selected from O, N and S.

The term "heteroarylene" as used herein, includes within its meaning divalent, single, polynuclear, conjugated and fused aromatic radicals having 1 to 12 atoms wherein 1 to 6 atoms are heteroatoms selected from O, N and S.

The term "heterocycloalkyl" as used herein, includes within its meaning monovalent, saturated, monocyclic, bicyclic, polycyclic or fused radicals wherein 1 to 5 atoms are heteroatoms selected from O, N or S.

The term "heterocycloalkylene" as used herein, includes within its meaning divalent, saturated, monocyclic, bicyclic, polycyclic or fused polycyclic radicals wherein 1 to 5 atoms are heteroatoms selected from O, N or S.

The term "heterocycloalkenyl" as used herein, includes within its meaning monovalent, saturated, monocyclic, bicyclic, polycyclic or fused polycyclic radicals having at least 1 double bond and wherein 1 to 5 atoms are heteroatoms selected from O, N or S.

The term "heterocycloalkenylene" as used herein, includes within its meaning divalent, saturated, monocyclic, bicyclic, polycyclic or fused polycyclic radicals having at least one double bond and wherein 1 to 5 atoms are heteroatoms selected from O, N or S.

The term "phenylarsonic acid" as used herein, is to be considered synonymous with "benzene arsonic acid".

Brief Description of the Drawings

Figure 1. GSAO triggered MTP opening by reacting with and perturbing ANT. A Structure of GSAO, GSAA, and GSCA. B GSAO triggered swelling of mitochondria. Swelling was measured by monitoring the associated decrease in light scattering at 520 nm over 60 min. The traces are representative of a minimum of three experiments on at least two different mitochondrial preparations. In part i, mitochondria were incubated with nil (●), 25 (○), 50 (▲), 100 (△) or 200 (■) μM GSAO. The positive control for pore opening was 150 μM Ca^{2+} and 6 mM Pi (□). In part ii, mitochondria were incubated with nil (●), 100 μM GSCA (△), 100 μM GSAA (▲) or 100 μM GSAO (■). The positive control for pore opening was 25 μM PAO (○). In part iii, mitochondria were incubated with 100 μM GSAO in the absence (■) or presence of 3 mM Mg^{2+} (▼), 100 μM EGTA (△), 10 μM BKA (▲), 5 μM CsA (▽), or 8 mM ADP (○). No treatment (●) is shown for comparison. C Increasing Ca^{2+} concentration sensitises the MPTP to GSAO. Mitochondria were incubated with 50 (●), 75 (○), 100 (■) or 200 μM (□) GSAO in the absence or presence of increasing Ca^{2+} concentration and the time for half-maximal swelling was measured. D Labelling of ANT with GSAO-B. Isolated rat mitochondria were labelled with GSAO-B in the absence (lane 1) or presence (lane 2) of a 4-fold molar excess of 2,3-dimercaptopropanol (DMP) and the biotin-labelled proteins were collected on streptavidin-agarose beads, resolved on SDS-PAGE and Western blotted for ANT. E GSAO competed for alkylation of Cys^{160} in ANT by eosine-maleimide. Rat sub-mitochondrial particles were labelled with eosin-5-maleimide in the absence (lane 1) or presence (lane 2) of GSAO and the eosin-labelled ANT was resolved on SDS-PAGE and detected by transillumination.

Figure 2. GSAO concentrated in mitochondria in viable cells. Confocal microscopy of BAE cells incubated with GSAO-F (i) and Mitotracker Red (ii), showing colocalisation of the compounds in the mitochondria of a single cell. GSAO-F did not stain cells when incubated with DMP (iii and iv). The pentavalent arsenical, GSAA-F, also did not stain cells (v and vi).

Figure 3. GSAO inhibited ATP production, increased superoxide levels and triggered mitochondrial depolarisation and apoptosis in proliferating, but not growth-quiescent, endothelial cells. A GSAO reduced the viability of proliferating, but not growth-quiescent, endothelial cells. In contrast, PAO was equally toxic to both types of cell. BAE cells were arrested for 24 h in medium containing 0.25% serum, then cultured for a further 48 h in medium containing 0.25% or 10% serum, with GSAO (i) or PAO (ii). Number of attached cells after 48 h treatment was measured. Results are mean ± SE of three experiments. B GSAO triggered mitochondrial depolarisation and induced apoptosis in BAE cells. Cells were cultured for 48 h in medium containing 10% serum and GSAO and then labelled with JC-1 or annexin V. The percent of cells positive for mitochondrial membrane

depolarisation (JC-1 green/red fluorescence) and induction of apoptosis (annexin V binding) was measured. The same result was observed in two different experiments. C GSAO inhibited ATP production in proliferating, but not growth-quiescent, endothelial cells. BAE cells were cultured for 24 h in medium containing 0.5% or 10% serum and then for a further 24 h in the presence of GSAO. In part i, cellular ATP levels were measured using a luciferin/luciferase assay. Results are mean ± SE of four experiments. In part ii, mitochondrial mass was measured from uptake of nonyl acridine orange (NAO). Results are mean ± range of two experiments. C GSAO increased the levels of superoxide (O_2^-) in proliferating, but not growth-quiescent, endothelial cells. BAE cells were cultured for 24 h in medium containing 0.5% or 10% serum and then for a further 24 h in the presence of GSAO. Cellular O_2^- levels were measured using dihydroethidium. Results are mean ± range of two experiments.

Figure 4. GSAO inhibited CAM angiogenesis and tumour angiogenesis and tumour growth in mice. A Photographs of CAM's after incubation with methylcellulose discs containing 10 µg of either GSAA (top) or GSAO (bottom) for 48 h. The dotted circle indicates the placement of the disc. B The number out of 5 zones positive for angiogenesis inhibition at 5, 10 or 50 µg of GSAO per pellet. GSAA did not inhibit CAM angiogenesis up to 50 µg per pellet. C-E SCID mice bearing ~0.1 g BxPC-3 (part C) or HT1080 (part D) tumours, or C57Bl6/J mice bearing ~0.1 g LLC tumours (part E), were randomised into two groups (n = 4) and treated *S.C.* with either GSAA or GSAO at 10 mg/kg/day in 0.2 mL of PBS containing 100 mM glycine. The data points are the mean ± SE of the tumour volumes. E Histological sections of the BxPC-3 tumours from the experiment shown in part C at day 31 of treatment with GSAA or GSAO were analysed for angiogenesis (CD31), proliferation (PCNA) and apoptosis (TUNEL).

Best Mode of Performing the Invention

The present invention relates to processes for identifying compounds which bind to ANT in mitochondria and selectively induce the MPT in proliferating cells, relative to non-proliferating cells.

ANT is a 30 kD protein that spans the inner mitochondrial membrane and is central to the MPTP (Crompton et al., 1988). There are three unpaired cysteines on the matrix side of ANT - Cys^{57}, Cys^{160} and Cys^{257}, (Halestrap et al., 1997). ANT activity is controlled by the binding of Ca^{2+}, cyclophilin D and adenine nucleotides. Cyclophilin D (Crompton et al., 1988) and adenine nucleotides (Haworth & Hunter, 1979a,b; Haworth & Hunter, 2000) bind to the matrix side of ANT, while the site(s) for interaction of Ca^{2+} has yet to be determined. There are several known regulators of the MPTP that appear to act by modulating binding of these three compounds. The primary trigger for opening of the MPTP is a rise in matrix Ca^{2+} concentration. Chelation of Ca^{2+}

with EGTA blocks pore opening. The specificity of the trigger site (presumably on ANT) for Ca^{2+} appears to be absolute as other divalent metal ions such as Mg^{2+} act as inhibitors (Haworth & Hunter, 1979a). Binding of cyclophilin D to ANT is necessary for pore opening at sub-millimolar Ca^{2+} concentrations (Halestrap et al., 2002). Cyclosporin A (CsA) blocks pore opening by binding to cyclophilin D and displacing it from ANT (Crompton et al., 1988). Matrix ADP is also an important modulator of pore opening that binds to ANT and decreases the sensitivity of the trigger site to Ca^{2+} (Haworth & Hunter, 1979a,b). Bonkrekic acid (BKA) also interacts with ANT and decreases sensitivity to Ca^{2+} (Hunter & Haworth, 1979).

Generally, the process according to this aspect of the invention comprises contacting a cell or cell extract with a candidate compound, determining whether the compound binds to ANT, and then determining whether the compound selectively induces the MPT in proliferating cells. Assessing whether a compound selectively induces the MPT in proliferating cells can be determined by comparing the effect of a compound identified as binding to ANT on the MPT in proliferating cells with the effect of the compound on the MPT in non-proliferating or growth quiescent cells.

Monitoring induction of MPT can be done using standard techniques known to those in the art. For example, spectrophotometrically monitoring the change in light scattering at a given wavelength, for differing concentrations of compound. A suitable wavelength is typically 520nm.

Detecting the uptake of a compound into the mitochondria of a cell can be carried out using standard techniques. For example, fluorescent labelled compounds can be used or a fluorophore can be attached to a compound. Examples of suitable fluorophores include fluorescein and Cy™5.5. Sub-cellular localisation of compounds can be detected by standard techniques including confocal fluorescence microscopy. Alternatively, compounds can be attached to other detectable groups, eg biotin, and detected by staining with streptavidin, such as Streptavidin-Alexa Fluor 488.

Mitochondrial transmembrane potential can be assessed using standard techniques, including, for example, dyes, such as JC-1, and stains, such as annexin V-FITC. By such techniques it is possible to determine whether cells have undergone the MPT and assess whether apoptosis has been induced.

Reactive oxygen species, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), are generated as a by-product of ATP production and play an important role as signalling intermediates in cellular proliferation, but at elevated concentrations they arrest proliferation and induce apoptosis by damaging lipids, proteins, DNA, and RNA (Zanetti et al., 2001). O_2^- is converted in the mitochondria to H_2O_2 by Mn superoxide dismutase (SOD), but can also be released from

mitochondria through anion channels, where it is dismuted to H_2O_2 by cytosolic Cu/Zn SOD (Vanden Hoek et al., 1998).

Another aspect of the invention relates to a process for identifying a compound which induces apoptosis in proliferating cells, wherein the process comprises contacting a cell or cell extract with a candidate compound, determining whether there is an increase in cellular superoxide anion (O_2^-) concentration, and then determining whether the compound selectively induces apoptosis in proliferating cells relative to non-proliferating or growth quiescent cells. Assessing whether a compound selectively induces apoptosis in proliferating cells can be readily determined by those skilled in the art by comparing the effect of the compound on cellular superoxide anion levels in proliferating cells with the effect in non-proliferating or growth quiescent cells.

Measurement of cellular superoxide levels can be carried out using assays well known to those skilled in the art.

A further aspect of the invention is a process for identifying a compound which is an inhibitor of angiogenesis, wherein the process comprises contacting a cell or cell extract with a candidate compound, determining whether there is an increase in cellular superoxide anion (O_2^-) concentration, and thereby determining whether the compound is an inhibitor of angiogenesis.

Angiogenesis refers to the sprouting of new capillaries from existing vessels and is driven by proliferating endothelial cells. Angiogenesis occurs during embryogenesis and in the adult (Carmeliet & Jain, 2001). In the normal adult mammal angiogenesis is confined to the female reproductive cycles, wound healing and several pathological situations. Angiogenesis is a key factor in diseases such as rheumatoid arthritis, psoriasis, diabetic retinopathy and cancer. Tumour expansion and metastasis is dependent on tumour angiogenesis (Hanahan & Folkman, 1996).

Therapeutic Applications

Perturbation of the MPT by compounds which bind to ANT can induce apoptosis. Further, reactive oxygen species such as superoxide anion (O_2^-) play an important role as signalling intermediates in cellular proliferation, but elevated concentrations can arrest proliferation and induce apoptosis. Accordingly, compounds which bind to ANT and selectively inhibit mitochondrial function, or compounds which increase cellular levels of superoxide anion, in proliferating cells compared to non-proliferating cells have the potential to be therapeutically useful for treating various diseases and conditions in vertebrates.

Examples of disorders and diseases may be grouped into broad categories such as the following: angiogenesis-dependent diseases, cellular proliferative diseases (e.g. psoriasis, IBD, malignancies, restenosis), inflammatory disorders, auto-immune diseases, blood vessel diseases,

thrombosis, cancer, neurodegenerative disorders (e.g. Alzheimer's disease, Parkinson's disease), myelodysplastic syndromes, ischaemia/repurfusion injury and organ transplant injury.

Typically, the cancer is selected from the group consisting of carcinogenic tumours, tumours of epithelial origin, such as colo-rectal cancer, breast cancer, lung cancer, head and neck tumours, hepatic cancer, pancreatic cancer, ovarian cancer, gastric cancer, brain cancer, bladder cancer, prostate cancer and urinary/genital tract cancer, mesenchymal tumours, such as sarcoma; and haemopoietic tumours such as B cell lymphoma.

Typically, the cancer is a haematological tumour. More typically, the cancer is a solid tumour.

Other potential therapeutic applications of the present invention include treatment of inflammatory disorders and/or auto-immune diseases, examples of which include the following: rheumatoid arthritis, seronegative arthritides and other inflammatory arthritides, systemic lupus erythematosus, polyarteritis and related syndromes, systemic sclerosis, Sjögren's syndrome and other inflammatory eye disease, mixed connective tissue disease, polymyositis and dermatomyositis, polymyalgia rheumatica and giant cell arteritis, inflammatory joint disease, non-inflammatory arthropathies and soft tissue rheumatism, algodystrophy.

Examples of blood vessel disease and thrombosis include the following: progression of atherosclerosis; cerebrovascular accidents such as transient ischaemic, completed stroke, and after carotid surgery; acute myocardial infarction (primary and secondary); angina; occlusion of coronary artery bypass graft; occlusion following percutaneous transluminal coronary angioplasty; occlusion following coronary stenting; vascular occlusion in peripheral arterial disease; venous thromboembolic disease following surgery, or during pregnancy, or during immobilisation.

Examples of small vessel disease include the following: glomerulonephritis; thrombotic thrombocytopenic purpura; the haemolytic uraemic syndrome; placental insufficiency and preeclampsia.

The present invention may also have applications in the treatment of vascular syndromes and myeloproliferative diseases.

The present invention may also find use in identifying compounds which prevent thrombosis formation in the following situations: artificial/prosthetic vascular shunts and grafts; prosthetic heart valves; cardiopulmonary bypass procedures; haemoperfusion and haemodialysis.

Typically, compounds identified in accordance with the invention may be used in combination with other known treatments, such as surgery and/or therapeutic agents, including chemotherapeutic or radiotherapeutics. For example, when used in the treatment of solid tumours, compounds identified in accordance with the present invention may be administered with chemotherapeutic agents such as: adriamycin, taxol, fluorouricil, melphalan, cisplatin, alpha

interferon, COMP (cyclophosphamide, vincristine, methotrexate and prednisone), etoposide, mBACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone), PROMACE/MOPP (prednisone, methotrexate (w/leucovin rescue), doxorubicin, cyclophosphamide, taxol, etoposide/mechlorethamine, vincristine, prednisone and procarbazine), vincristine, vinblastine, angioinhibins, TNP-470, pentosan polysulfate, platelet factor 4, angiostatin, LM-609, SU-101, CM-101, Techgalan, thalidomide, SP-PG and the like. Other chemotherapeutic agents include alkylating agents such as nitrogen mustards including mechloethamine, melphan, chlorambucil, cyclophosphamide and ifosfamide; nitrosoureas including carmustine, lomustine, semustine and streptozocin; alkyl sulfonates including busulfan; triazines including dacarbazine; ethyenimines including thiotepa and hexamethylmelamine; folic acid analogues including methotrexate; pyrimidine analogues including 5-fluorouracil, cytosine arabinoside; purine analogues including 6-mercaptopurine and 6-thioguanine; antitumour antibiotics including actinomycin D; the anthracyclines including doxorubicin, bleomycin, mitomycin C and methramycin; hormones and hormone antagonists including tamoxifen and cortiosteroids and miscellaneous agents including cisplatin and brequinar.

Typically, the physiological system to be treated in accordance with the present invention (e.g. the hepatic system, pancreatic system) may be isolated by or during surgery prior to administration of the system of the invention.

Single or multiple administrations of the compounds or pharmaceutical compositions can be carried out with dose levels and pattern being selected by the treating physician. Regardless, the compounds or pharmaceutical compositions identified according to the present invention should provide a quantity of the compound sufficient to effectively treat the patient.

One skilled in the art would be able, by routine experimentation, to determine an effective, non-toxic amount of the compounds or pharmaceutical compositions used in accordance with the invention which would be required to detect apoptotic cells and/or treat or prevent the disorders and diseases. Generally, an effective dosage is expected to be in the range of about 0.0001mg to about 1000mg per kg body weight per 24 hours; typically, about 0.001mg to about 750mg per kg body weight per 24 hours; about 0.01mg to about 500mg per kg body weight per 24 hours; about 0.1mg to about 500mg per kg body weight per 24 hours; about 0.1mg to about 250mg per kg body weight per 24 hours; about 1.0mg to about 250mg per kg body weight per 24 hours. More typically, an effective dose range is expected to be in the range about 1.0mg to about 200mg per kg body weight per 24 hours; about 1.0mg to about 100mg per kg body weight per 24 hours; about 1.0mg to about 50mg per kg body weight per 24 hours; about 1.0mg to about 25mg per kg body weight per

24 hours, about 5.0mg to about 50mg per kg body weight per 24 hours, about 5.0mg to about 20mg per kg body weight per 24 hours, about 5.0mg to about 15mg per kg body weight per 24 hours.

Alternatively, an effective dosage may be up to about 500mg/m^2. Generally, an effective dosage is expected to be in the range of about 25 to about 500mg/m^2, preferably about 25 to about 350mg/m^2, more preferably about 25 to about 300mg/m^2, still more preferably about 25 to about 250mg/m^2, even more preferably about 50 to about 250mg/m^2, and still even more preferably about 75 to about 150mg/m^2.

In relation to GSAO, an effective dosage is in the range of about 0.0001 mg to about 100 mg GSAO per kg body weight per 24 hours, preferably about 0.001 mg to about 100 mg GSAO per kg body weight per 24 hours, more preferably about 0.01 mg to about 50 mg GSAO per kg body weight per 24 hours, even more preferably about 0.1 mg to about 20 mg GSAO per kg body weight per 24 hours, even more preferably still about 0.1 mg to about 10 mg GSAO per kg body weight per 24 hours.

In relation to an additional active agent used together with a compound identified in accordance with the present invention, an effective dosage is in the range of about 0.0001 mg to about 100 mg agent per kg body weight per 24 hours, preferably about 0.001 mg to about 100 mg agent per kg body weight per 24 hours, more preferably about 0.01 mg to about 50 mg agent per kg body weight per 24 hours, even more preferably about 0.1 mg to about 20 mg agent per kg body weight per 24 hours, even more preferably still about 0.1 mg to about 10 mg agent per kg body weight per 24 hours.

Typically the compound(s) would be administered for the duration of the condition.

Further, it will be apparent to one of ordinary skill in the art that the optimal quantity and spacing of individual dosages of a compound used in accordance with the present invention will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the nature of the particular vertebrate being treated. Also, such optimum conditions can be determined by conventional techniques.

It will also be apparent to one of ordinary skill in the art that the optimal course of treatment, such as, the number of doses of the compound given per day for a defined number of days, can be ascertained by those skilled in the art using conventional course of treatment determination tests.

Whilst the compounds identified in accordance with the process of the present invention may be administered alone, it is generally preferable that the compound be administered as a pharmaceutical composition/formulation. In general pharmaceutical formulations may be prepared according to methods which are known to those of ordinary skill in the art and accordingly may include a pharmaceutically acceptable carrier, diluent and/or adjuvant.

The invention will now be illustrated with reference to the following examples.

Example 1

Synthesis of Compounds

Methods

Synthesis of GSAO

BRAA was prepared as described above, and was then converted to 4-(*N*-(bromoacetyl)amino)benzenearsonous acid (BRAO) using the method described in Donoghue et al (2000). The procedure for the conversion of BRAO to GSAO is a modification of that described in Donoghue et al. (2000), as follows. Reduced glutathione (16 g, 52 mmol) was dissolved in deoxygenated water (1 L) under a nitrogen atmosphere (deoxygenated water was prepared by boiling followed by cooling to room temperature, all the time under an atmosphere of nitrogen). BRAO (25 g, 77 mmol) was suspended in the solution, and vigorous stirring of the mixture was applied until the undissolved BRAO had lost all tendency to float, at which point the rate of stirring was decreased. Triethylamine (16 mL, 11.6 g, 115 mmol) was added, and the mixture was stirred for at least 18 hours, all the time under nitrogen. Some colourless solid was removed by rapid vacuum filtration, and the filtrate was concentrated to a viscous gel on a rotary evaporator. The gel was diluted with ethanol (250 mL), the rate of stirring was increased, and then acetone (250 mL) was added, with formation of a white solid. After stirring for 2 hours under nitrogen, the solid was collected by vacuum filtration, and dried at room temperature under vacuum to a constant weight. Analysis of the material by ^{1}H-NMR allowed the amount of triethylamine to be determined. An equivalent amount of sodium hydroxide (ie. 1 mmol NaOH per mmol triethylamine) was added in the form of a 10 M aqueous solution to a solution of the material in the minimum amount of deoxygenated water. The solution was stirred under nitrogen for 2 hours, and GSAO (as the sodium salt) was obtained by removal of the solvent on a rotary evaporator. The GSAO was characterised by the following methods: ^{1}H-NMR (D_2O, 400 MHz), ^{13}C-NMR (D_2O, 100 MHz), HPLC, and infrared spectroscopy (nujol mull). The elemental analysis was consistent with a composition of GSAO.2H_2O, and the mass spectrum gave the parent molecular ion at 549.1 m.u. $[GSAO+H]^+$. The purity of GSAO was > 94% by HPLC.

Synthesis of GSAA

BRAA was prepared by a modification of the method described in Donoghue et al (2000). *p*-Arsanilic acid (20.6 g, 95 mmol) was added in portions to a solution of sodium carbonate (20 g, 189 mmol) in water (200 mL). When all solids had dissolved, the solution was found to be pH 10, and was chilled at 4°C for 2 hours. Bromoacetyl bromide (15 mL, 173 mmol) in dry dichloromethane (35

mL) was added in two portions, each addition followed by vigorous shaking for 2 to 3 min. The mixture was allowed to stand for a few minutes, and the lower organic layer was drained off. 4-(*N*-(Bromoacetyl)amino)benzenearsonic acid (BRAA) was precipitated by acidification of the solution to about pH 2–3 with the dropwise addition of 98% sulfuric acid, collected by vacuum filtration, and dried, giving BRAA as a white solid.

BRAA (3.38 g, 10 mmol), reduced glutathione (3.23 g, 10.5 mmol) and sodium bicarbonate (3.15 g, 37.5 mmol) were mixed together, and the solid mixture was dissolved in portions in 0.5 M bicarbonate buffer (100 mL). The clear solution was found to be pH 9, and was thoroughly mixed and left overnight at room temperature. On the following day, the solution was acidified to neutral pH with the dropwise addition of 32% hydrochloric acid, and the product precipitated from absolute ethanol (1 L) by dropwise addition of the acidified solution to the well-stirred alcohol. The mixture was stirred at room temperature for 1 hour, and then left for 3 hours until the precipitate had settled. The clear ethanolic solution was decanted until ~300 mL were left, and then this was swirled and centrifuged at 2000g for 5 min. The product 4-(*N*-((*S*-glutathionyl)acetyl)amino)benzenearsonic acid (GSAA) was washed by re-suspension in fresh absolute ethanol and centrifuged again. The washing was repeated two more times, and the final suspension was dried to a white solid, GSAA, by rotary evaporation. The GSAA was characterised by ^{1}H-NMR (D_2O, 300 MHz) and ^{13}C-NMR (D_2O, 75 MHz) and has a molecular weight of 564.

Synthesis of GSCA

GSCA was prepared in a similar manner to GSAA, using 4-aminobenzoic acid instead of *p*-arsanilic acid as follows. A chilled alkaline solution of 4-aminobenzoic acid (13 g, 95 mmol) was prepared as described above for *p*-arsanilic acid, and reacted in an identical manner with bromoacetyl bromide. The lower organic layer was drained off, and 4-(*N*-(bromoacetyl)amino)-benzoic acid (BRCA) precipitated directly from solution by itself. Following the rest of the procedure for GSAA exactly using BRCA (2.58 g, 10 mmol) in place of BRAA gave GSCA as a white solid after rotary evaporation. The GSCA was characterised by ^{1}H-NMR (D_2O, 300 MHz) and ^{13}C-NMR (D_2O, 75 MHz) and the disodium salt has a molecular weight of 528.

Synthesis of biotin and fluorescein derivatives of GSAO and GSAA

GSAO-B was produced as described by Donoghue et al. (2000) and has a molecular weight of 1001. A solution of fluorescein-5-EX succinimidyl ester (Molecular Probes, Eugene, OR) (2.4 mg, 4.1 µmol) in DMSO (240 µL) was added to GSAO or GSAA (33.8 mM) in Mes buffer, pH 5.5 (5 mM, 473 µL), the mixture was diluted with bicarbonate buffer, pH 9 (0.5 M, 3.287 mL) and allowed to stand at room temperature for 80 min. The reaction was then diluted with glycine (100 mM) in PBS (4 mL), and allowed to stand at room temperature overnight. The final solution contained

trivalent arsenical (2.00 mM) and glycine (50 mM). The molar ratio of fluorescein-5X to GSAO or GSAA was ~1.5:1. The molecular weights of GSAO- and GSAA-fluorescein (GSAO-F and GSAA-F) are 1024 and 1040, respectively.

Assay of arsenical concentration

The concentrations of GSAO, GSAO-B and GSAO-F were measured by titrating with dimercaptopropanol and calculating the remaining free thiols with 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma, St. Louis, MO) (Donoghue et al., 2000). The conjugates were sterile filtered and stored at 4°C in the dark until use. There was no significant loss in the active concentration of stock solutions of the arsenicals for at least a week when stored under these conditions.

Example 2

GSAO triggered MTP opening by reacting with and perturbing ANT

Methods

Mitochondria swelling assay

Mitochondria were isolated from the livers of ~250g male Wistar rats using differential centrifugation as described previously (Schnaitman & Greenawalt, 1968). The final mitochondrial pellet was resuspended in 3 mM HEPES-KOH, pH 7.0 buffer containing 213 mM mannitol, 71 mM sucrose and 10 mM sodium succinate at a concentration of 30 mg of protein per mL. MPT induction was assessed spectrophotometrically by suspending the liver mitochondria at 0.5 mg of protein per mL at 25°C in 3 mM HEPES-KOH, pH 7.0 buffer containing 75 mM mannitol, 250 mM sucrose, 10 mM sodium succinate, and 2 μM rotenone. All buffer components were from Sigma, St. Louis, MO. The arsenical derivatives and GSCA were dissolved in phosphate-buffered saline (PBS) containing 100 mM glycine. ANT-binding compounds employed were CsA, BKA and ADP (Sigma, St. Louis, MO). Reagent concentrations are indicated in the figure legends. Swelling was measured by monitoring the associated decrease in light scattering at 520 nm using a Molecular Devices Thermomax Plus (Palo Alto, CA) microplate reader.

Binding of GSAO to ANT

Rat liver mitochondria were suspended at 1 mg of protein per mL at 25°C in 3 mM HEPES-KOH, pH 7.0 buffer containing 213 mM mannitol, 71 mM sucrose and 10 mM sodium succinate and incubated with 100 μM GSAO-B in absence or presence of 400 μM 2,3-dimercaptopropanol (Fluka, Buchs, SG, Switzerland) at room temperature on a rotating wheel for 1 h. The labelled mitochondria were washed three times with PBS and sonicated in 0.3 mL of ice-cold 25 mM Tris, pH 7.4 buffer containing 140 mM NaCl, 2.7 mM KCl, 0.5% Triton X-100, 0.05% Tween-20, 10 μM leupeptin, 10 μM aprotinin, 50 μg mL^{-1} 4,2-(aminoethyl)-benzene sulfonyl fluoride and 5 mM EDTA.

Lysate was clarified by centrifugation at 18000g for 10 min at 4°C and incubated with 30 µL of streptavidin-dynabeads (Dynal, Oslo, Norway) for 60 min at 4°C on a rotating wheel to isolate the biotin-labelled proteins. The beads were washed 5 times with sonication buffer and the biotin-labelled proteins were released from the beads by boiling in 30 µl of SDS-Laemmli buffer for 2 minutes. Samples were resolved on 8-16% gradient iGels under reducing conditions and transferred to PVDF membrane. Proteins were detected by Western blot using a 1:500 dilution of goat anti-human ANT polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 1:2000 dilution of rabbit anti-goat peroxidase-conjugated antibodies (Dako, Carpinteria, California).

Labelling of ANT with eosin-5-maleimide

Rat liver sub-mitochondrial particles were prepared by sonication and differential centrifugation according to Majima et al. (1998). The particles were suspended at 20 mg of protein per mL in 10 mM Tris, pH 7.2 buffer containing 250 mM sucrose and 0.2 mM EDTA and incubated without or with 200 nmol GSAO per mg of protein for 10 min at 25°C, followed by 20 nmol of eosin-5-maleimide per mg of protein for 1 min at 0°C in the dark. The labelling was terminated by addition of 10 µmol of dithiothreitol per mg of protein. The proteins were resolved on 4-20% gradient iGels and the eosin-labelled ANT was visualised by UV transillumination using a Fluor-S™ MultiImager (Bio-Rad, Hercules, CA).

Results

GSAO (Fig. 1A) triggered swelling of isolated rat liver mitochondria (Fig. 1Bi). The rate of swelling increased with increasing GSAO concentration and time of incubation. The trivalent arsenical moiety of GSAO was responsible for this activity as the corresponding pentavalent arsenical (4-(*N*-((*S*-glutathionyl)acetyl)amino)benzenearsonic acid, GSAA) or carboxylic acid (4-(*N*-((*S*-glutathionyl)acetyl)amino)benzoic acid, GSCA) compounds (Fig. 1A) were without effect (Fig. 1Bii). Positive controls for pore opening were Ca^{2+} and Pi ions (Fig. 1Bi) and PAO (Fig. 1Bii).

ANT activity is controlled by the binding of Ca^{2+}, cyclophilin D and adenine nucleotides. Cyclophilin D (Crompton et al., 1988) and adenine nucleotides (Haworth & Hunter, 1979a,b) bind to the matrix side of ANT, while the site(s) for interaction of Ca^{2+} has yet to be determined. The primary trigger for opening of the MPTP is a rise in matrix Ca^{2+} concentration. Chelation of Ca^{2+} with EGTA blocks pore opening. The specificity of the trigger site for Ca^{2+} appears to be absolute as other divalent metal ions such as Mg^{2+} act as inhibitors (Haworth & Hunter, 1979a). Binding of cyclophilin D to ANT is necessary for pore opening at sub-millimolar Ca^{2+} concentrations (Halestrap et al., 2002). Cyclosporin A (CsA) blocks pore opening by binding to cyclophilin D and displacing it from ANT (Crompton et al., 1988). Matrix ADP is also an important modulator of pore opening that

binds to ANT and decreases the sensitivity of the trigger site to Ca^{2+} (Haworth & Hunter, 1979a,b). Bonkrekic acid (BKA) also interacts with ANT and decreases sensitivity to Ca^{2+} (Hunter & Haworth, 1979).

Mg^{2+}, EGTA, CsA, ADP, and BKA all blocked the effect of GSAO on pore opening (Fig. 1Biii). These observations are consistent with a specific effect of GSAO on pore opening and support ANT as its target.

The half-time for GSAO mediated swelling of the mitochondria was reduced by added Ca^{2+} (Fig. 1C). This result is consistent with GSAO cross-linking two of the three cysteine residues on ANT that protrude into the mitochondrial matrix.

Direct interaction of ANT with a biotin-tagged GSAO (4-(*N*-(*S*-(*N*-(6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoyl)-glutathionyl)acetyl)amino) phenylarsenoxide, GSAO-B) is shown in Fig. 1D. The labeling of ANT with GSAO-B was specific as it was competed for by a 4-fold molar excess of the small synthetic dithiol, dimercaptopropanol. Eosine-maleimide alkylates Cys^{160} in ANT (Majima et al., 1998) and blocks ADP binding (Halestrap et al., 1997). GSAO competed for eosine-maleimide alkylation of ANT Cys^{160} (Fig. 1E), which implied that GSAO cross-links Cys^{160} and Cys^{257}.

Example 3

GSAO concentrated in mitochondria in viable cells

Methods

Cell culture

Bovine aortic endothelial (BAE) cells (ATCC, Rockville, MD) were cultured in DMEM supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, and 5 $U.mL^{-1}$ penicillin/streptomycin (Gibco, Gaithersburg, MD). The human microvascular endothelial cell line, HMEC-1 (Ades et al., 1992), was cultured in MCDB131 medium (Gibco) supplemented with 10% FCS, 2 mM L-glutamine, 5 $U.mL^{-1}$ penicillin/streptomycin, 10 $ng.mL^{-1}$ epidermal growth factor (Sigma, St. Louis, MO) and 1 $\mu g.mL^{-1}$ hydrocortisone (Sigma). Cell culture plasticware was purchased from Corning Costar (Corning, NY). Trypsin/EDTA solution was from Gibco.

Immunocytochemistry and confocal microscopy

BAE cells were seeded at a density of 10^4 cells per well into 8-well Labtek glass chamber slides (Nunc, Naperville, IL) and allowed to adhere overnight. The cells were washed once and the medium was replaced with Hanks Balanced Salt Solution (HBSS) (Gibco). The cells were then incubated for 1 h with 50 µM GSAO-F, 50 µM GSAO-F and 250 µM dimercaptopropanol, or 50 µM GSAA-F. All cells were counterstained with 100 nM Mitotracker™ Red CMXRos (Molecular

Probes, Eugene, OR). The cells were then washed three times with HBSS, fixed for 10 min with 80% acetone and 20% methanol, and washed three times with HBSS. Slides were mounted in VectaShield antifade agent (Vector Laboratories, Burlingame, CA) and sealed with nail polish. Images were captured using a Leica DM IRB inverted microscope and confocal system, with Leica confocal software.

A striking feature of GSAO was that it rapidly localised to mitochondria in viable bovine aortic (BAE) (Fig. 2) and human dermal microvascular (HMEC-1) (data not shown) endothelial cells. GSAO was conjugated to fluorescein (GSAO-F) and its sub-cellular localisation was determined by confocal fluorescence microscopy. Mitochondrial staining was confirmed by colocalisation of GSAO-F with the red-fluorescent Mitotracker probe, which accumulates in the mitochondria of actively respiring cells (Fig. 2ii, iv and vi). The mitochondrial accumulation of GSAO-F was specific for the dithiol reactivity of GSAO as it was abolished in the presence of a five-fold molar excess of the small synthetic dithiol, dimercaptopropanol (Figure 2iii), and the corresponding pentavalent arsenical, GSAA-F, did not stain the cells (Figure 2v). Mitochondrial localisation was also seen in BAE cells incubated with GSAO-B, followed by staining with streptavidin-Alexa Fluor 488 (data not shown).

ANT is abundant in the inner mitochondrial membrane. Sequestration of GSAO on the matrix face of ANT would drive entropic import of GSAO into the matrix. This mechanism is supported by the finding that GSAA, which has effectively the same charge and size as GSAO, did not react with ANT or accumulate in mitochondria.

Example 4

GSAO inhibited ATP production, increased superoxide levels and triggered mitochondrial depolarisation and apoptosis in proliferating, but not growth-quiescent, endothelial cells

Methods

ATP assays

BAE cells in six-well culture plates were arrested for 24 h in 0.5% serum or left in 10% serum, then treated for 24 h with 0, 60 or 150 μM GSAO. The cells were washed once then resuspended in 0.4 mL PBS containing 0.3% bovine serum albumin. A 50 μl sample of cells was mixed with 50 μL water and 100 μL ATP releasing agent (Sigma) and ATP concentration was measured using a luciferin/luciferase ATP assay mix (Sigma). Light units were converted to ATP concentrations using an ATP standard in place of the 50 μL water. Cell number was counted using a Beckman Coulter Counter and mol of ATP was expressed per million cells.

Cell proliferation assays

BAE or HMEC-1 cells were seeded at a density of 10^4 cells per cell into 96 well plates and allowed to adhere overnight and then treated as indicated. For experiments involving arrest of cell proliferation in 0.25% serum, the cells were seeded in medium containing 10% serum, allowed to adhere overnight, then washed with PBS and arrested for 24 h in medium containing 0.25% serum. Attached cells remaining after treatments were measured using methylene blue (Oliver et al., 1989).

Superoxide, mitochondrial membrane potential, mitochondrial mass and apoptosis assays

For measurements of superoxide levels with dihydroethidine, mitochondrial membrane potential with JC-1, mitochondrial mass with nonyl acridine orange, and apoptosis with annexin V, proliferating or growth-quiescent BAE cells were treated for 24 or 48 h with GSAO in six-well culture plates, then detached with trypsin/EDTA, washed once, and resuspended at 2 x 10^5 cells per mL in serum-free medium. Cells were then incubated for 15 min at room temperature with dihydroethidine (5 μM, Molecular Probes) or 30 min at room temperature with JC-1 (0.5 μg.mL^{-1}, Molecular Probes), annexin V-FITC (50 μl.mL^{-1}) (Pharmingen, La Jolla, CA, USA), or acridine orange 10-nonyl bromide (5 μM, Molecular Probes). Cells were then washed once, resuspended in 0.5 mL serum-free medium, transferred to ice, and fluorescence was quantitated immediately by flow cytometry.

Results

GSAO concentrated in mitochondria in viable cells and perturbed mitochondrial function and cell viability. Proliferating cells having a greater mitochondrial mass and respiration than growth-quiescent cells. To test whether GSAO might have selective effects on proliferating cells, proliferating or growth-quiescent BAE cells were incubated for 48 h with increasing concentrations of GSAO.

The GSAO IC_{50} for reduction in viability of proliferating BAE cells was ~75 μM (Fig. 3Ai), whereas the compound had little effect on growth-quiescent cells. The control compounds, GSAA and GSCA, had no significant effect on the viability of proliferating BAE cells at concentrations up to 1.0 mM (data not shown). The effect of GSAO on proliferating cell viability was time-dependent. The GSAO IC_{50} decreased from ~75 μM to ~25 μM when the incubation time increased from 48 to 72 h (data not shown). PAO, in contrast, was equally toxic to both proliferating and growth-quiescent BAE cells with an IC_{50} of ~200 nM (Fig. 3A(ii)). The same results were observed with HMEC-1 cells (data not shown). GSAO's effect on proliferating endothelial cell viability was closely associated with loss of mitochondrial transmembrane potential and apoptosis (Fig. 3B). These

parameters were measured using the JC-1 dye and FITC-conjugated annexin V, respectively. The ratio of distribution of JC-1 between the cytosol (green fluorescence) and mitochondria (red fluorescence) reflects mitochondrial transmembrane potential (Smiley et al., 1991) while annexin V binds to phosphatidylserine exposed on the surface of apoptotic cells.

The binding of GSAO to ANT in proliferating endothelial cells affected ATP production. The ATP content of proliferating BAE cells was approximately twice that of growth-quiescent cells and incubation with 150 μM GSAO for 24 h reduced ATP levels in proliferating cells to that of growth-quiescent cells (Fig. 3Ci). This result mirrors the selective effects of GSAO on proliferating cell viability (Fig. 3Ai). To confirm that the decreased cellular ATP was due to an effect of GSAO on mitochondrial ATP synthesis rather than mitochondrial biogenesis, mitochondrial mass was measured using nonyl acridine orange. This dye binds to cardiolipin in the mitochondrial membrane (Petit et al., 1992). The mitochondrial mass of proliferating cells was approximately twice that of growth-quiescent cells, which was unchanged by GSAO treatment (Fig. 3Cii).

At high concentrations, reactive oxygen species such as superoxide anion (O_2^-) can arrest cellular proliferation and induce apoptosis. To test the effect of GSAO on cellular levels of O_2^-, BAE cells were treated for 24 h with GSAO and the cellular levels of O_2^- and H_2O_2 were measured using the dyes dihydroethidium and CM-H_2-DCFDA, respectively. Cellular levels of O_2^- increased linearly with GSAO concentration in proliferating, but not growth-quiescent, BAE cells (Fig. 3D). This effect of GSAO was not due to inhibition of SOD activity as there was only a small decline in H_2O_2 levels. For instance, treatment with 120 μM GSAO for 24 h resulted in a 30% decrease in H_2O_2, but a 100% increase in the level of O_2^-. This finding was confirmed using two-channel flow cytometric analysis of cellular O_2^- and H_2O_2 levels. O_2^- levels were increased in the majority of cells without a corresponding decrease in H_2O_2 (data not shown). In contrast, GSAA had no effect on O_2^- or H_2O_2 levels.

Example 5

GSAO Inhibited angiogenesis in the chick chorioallantoic membrane (CAM)

Methods

Fertilised 3 day-old white Leghorn eggs (Spafas, Norwich, CT) were cracked, the embryos with intact yolks placed in 20 x 100 mm petri dishes and incubated for 3 days at 37°C and 3% CO_2 (Folkman, 1985). Methylcellulose (Fisher Scientific, Fair Lawn, NJ) discs containing 5, 10 or 50 μg of either GSAA or GSAO were then applied to the CAM of individual embryos and incubated for 48 h at 37°C and 3% CO_2. The discs were made by desiccation of GSAA or GSAO in 10 μl of 0.45% methycellulose on teflon rods. The CAMs were observed using a stereomicroscope and scored for

no obvious affect or inhibition of CAM angiogenesis as defined by avascular zones. On some occasions CAM blood vessels were injected with India ink and photographed.

Results

In view of the ability of GSAO to selectively kill proliferating, but not growth-quiescent, endothelial cells *in vitro*, GSAO was tested to determine whether it was an effective inhibitor of angiogenesis *in vivo*.

The chick chorioallantoic membrane (CAM) assay has been used for the detection and analysis of angiogenesis inhibition (Nguyen et al., 1994). GSAO inhibited CAM angiogenesis in a concentration-dependent manner (Fig. 4A). Angiogenesis inhibition was defined as avascular zones 48 h after implantation of methylcellulose pellets containing GSAO on the 6-day CAM (Fig. 4Ai). GSAA up to 50 μg per pellet had no affects on CAM angiogenesis. We next tested whether GSAO would inhibit tumour angiogenesis and tumour growth in mice.

Example 6

GSAO inhibited tumour angiogenesis and tumour growth in mice

Methods

Primary tumour growth assays

Female 7 to 9 week old SCID or C57Bl6/J mice were used (Massachusetts General Hospital, Boston, MA). Mice were held in groups of 3 to 5 at a 12 hour day and night cycle and were given animal chow and water *ad libidum*. SCID or C57Bl6/J mice were anaesthetised by inhalation of isoflurane, the dorsal skin shaved and cleaned with ethanol, and a suspension of 2.5×10^6 BxPC-3, HT1080 or LLC cells in 0.2 mL of PBS, or saline for LLC cells, was injected S.C. in the proximal midline. LLC cells were prepared according to O'Reilly et al. (1997). Tumour volume was calculated using the relationship, $a.b^2.0.52$, where a is the longest and b the shortest diameter.

Immunohistochemistry

Tumours were fixed in Buffered Formalde-Fresh (Fisher Scientific, Fair Lawn, NJ), embedded in paraffin and 5 μm thick sections were cut and placed on glass slides. Sections were stained with haematoxylin and eosin or for CD31, PCNA (Holmgren et al., 1995) or fragmented DNA (Gavrielli et al., 1992). Microvessels were counted in 3 tumours, including the smallest and largest, from the control and treatment groups and the density was graded in the most active areas of neovascularisation according to Weidner et al. (1991). The proliferative index was estimated by the percentage of cells scored under 400× magnification. A minimum of 1000 cells was counted in

two separate sections. The apoptotic index was estimated by the percentage of cells scored under 400x magnification. A minimum of 1500 cells was counted in two separate sections.

Results

The growth of both human and murine primary tumours in immunocompromised and immunocompetent mice, respectively, was markedly suppressed by systemic administration of GSAO. Treatment of SCID mice bearing BxPC-3 (Fig. 4C) or HT1080 (Fig. 4D) tumours, or C57Bl6/J mice bearing LLC tumours (Fig. 4E), by subcutaneous administration of 10 mg/kg/day GSAO at a site remote from the tumour resulted in >90%, ~70% and ~50% inhibition of the rate of tumour growth, respectively. Administration of control GSAA caused <20% inhibition of the rate of tumour growth in all experiments when compared to administration of vehicle alone (data not shown). There was no apparent adverse affects of administration of either GSAO or GSAA to either SCID or C57Bl6/J mice. The average mice weights of the GSAO and GSAA treatment groups over the course of the experiments were the same, and there was no apparent macroscopic differences and no morphological changes in the heart, lungs, liver, kidneys, and spleen of GSAO or GSAA-treated mice (data not shown).

Immunohistochemical analysis of the tumours from the experiment described in Fig. 4B indicated a significant reduction in blood vessel density in the GSAO-treated tumours ($p < 0.001$) (Fig. 4F). The proliferative indices of the GSAA- and GSAO-treated tumours were the same, while there was a significant increase in the apoptotic indices of GSAO- versus GSAA-treated tumours ($p=0.05$) (Fig. 4F). Inhibition of tumour angiogenesis has been associated with an increase in apoptosis of the tumour cells (O'Reilly et al., 1997). The high rate of apoptosis is thought to balance the high proliferative rate of the tumour cells resulting in no net gain in tumour size (Holmgren et al., 1995).

References

Ades, E. W., Candal, F. J., Swerlick, R. A., George, V. G., Summers, S., Bosse, D. C., and Lawley, T. J. HMEC-1: Establishment of an immortalized human microvascular endothelial cell line. J. Invest. Dermatol. 99, 683-690, 1992.

Carmeliet, P. and Jain, R. K. Angiogenesis in cancer and other diseases. Nature 407, 249-257, 2000.

Crompton, M. The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 341, 233-249, 1999.

Crompton, M., Costi, A. and Hayat, L. Evidence for the presence of a reversible Ca^{2+}-dependent pore activated by oxidative stress in heart mitochondria. Biochem. J. 245, 915-918, 1987.

Crompton, M., Ellinger, H. and Costi, A. Inhibition by cyclosporin A of a Ca^{2+}-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem. J. 255, 357-360, 1988.

Donoghue, N., Yam, P. T. W., Jiang, X., and Hogg, P. J. Presence of closely spaced protein thiols on the surface of mammalian cells. Protein Sci. 9, 2436-2445, 2000.

Folkman, J. Angiogenesis and its inhibitors. In Important Advances in Oncology, DeVita, V.T., Hellman, S., and Rosenberg, S., eds. (J.B. Lippincott Company, PD) pp. 42-62, 1985.

Halestrap, A.P., McStay, G.P., Clarke, S.J. The permeability transition pore complex: another view. Biochimie 84, 153-66, 2002.

Halestrap, A.P., Woodfield, K.Y. and Connern, C.P. Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. J. Biol. Chem. 272, 3346-3354, 1997.

Hanahan, D. and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumourigenesis. Cell 86, 353-364, 1996.

Haworth, R.A. and Hunter, D.R. Control of the mitochondrial permeability transition pore by high-affinity ADP binding at the ADP/ATP translocase in permeabilized mitochondria. J. Bioenerg. Biomembr. 32, 91-96, 2000.

Haworth, R.A. and Hunter, D.R. The Ca^{2+}-induced membrane transition in mitochondria. II. Nature of the Ca^{2+} trigger site. Arch. Biochem. Biophys. 195, 460-467, 1979.

Holmgren, L., O'Reilly, M.S., and Folkman, J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nature Med. 1, 149-153, 1995.

Hunter, D.R. and Haworth, R.A. The Ca^{2+}-induced membrane transition in mitochondria. I. The protective mechanisms. Arch. Biochem. Biophys. 195, 453-459, 1979.

Majima, E., Yamaguchi, N., Chuman, H., Shinohara, Y., Ishida, M., Goto, S. and Terada, H. Binding of the fluorescein derivative eosin Y to the mitochondrial ADP/ATP carrier: characterization of the adenine nucleotide binding site. Biochemistry 37, 424-432, 1998.

Nguyen, M., Shing, Y., and Folkman, J. Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane. Microvascular Res. 47, 31-40, 1994.

Oliver, M. H., Harrison, N. K., Bishop, J. E., Cole, P. J., and Laurent, G. J. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J. Cell Sci. 92, 513-518, 1989.

O'Reilly, M.S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W.S., Flynn, E., Birkhead, J.R., Olsen, B.R., and Folkman J. Endostatin: an endogenous inhibitor of angiogenesis and tumour growth. Cell 88, 277-285, 1997.

Petit, J.M., Maftah, A., Ratinaud, M.H., and Julien, R. 10N-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria. Eur. J. Biochem. 209, 267-273, 1992.

Schnaitman, C. and Greenawalt, J.W. Enzymatic properties of the inner and outer membranes of rat liver mitochondria. J. Cell Biol. 38, 158-75, 1968.

Smiley, S.T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T.W., Steele, G.D., and Chen, L.B. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. Proc. Natl. Acad. Sci. USA 88, 3671-3675, 1991.

Vanden Hoek, T.L., Becker, L.B., Shao, Z., Li, C., and Schumacker, P.T. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. J. Biol. Chem. 29, 18092-18098, 1998.

Zanetti, M., Zwacka, R.M., Engelhardt, J.F., Katusic, Z.S., and O'Brien, T. Superoxide anions and endothelial cell proliferation in normoglycemia and hyperglycemia. Arterioscler. Thromb. Vasc. Biol. 21, 195-200, 2001.

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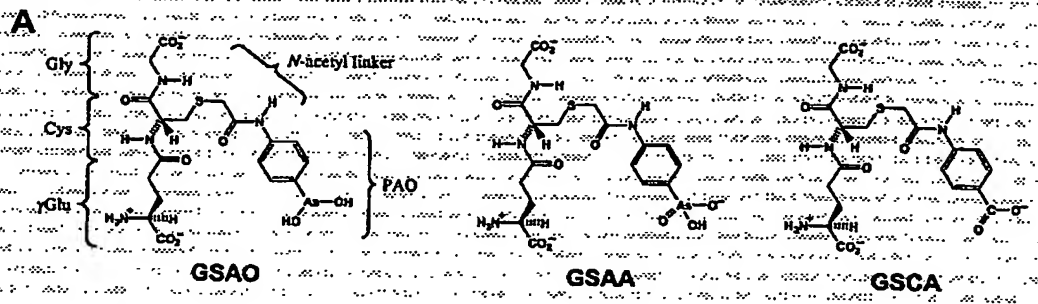
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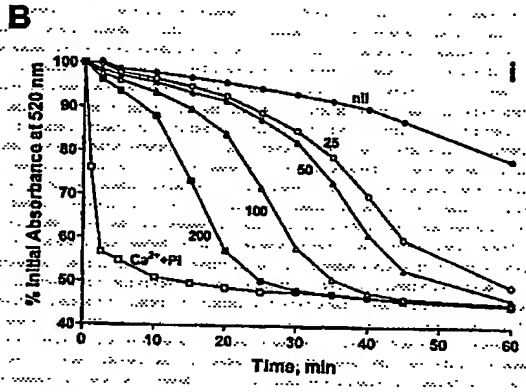
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50
100
200
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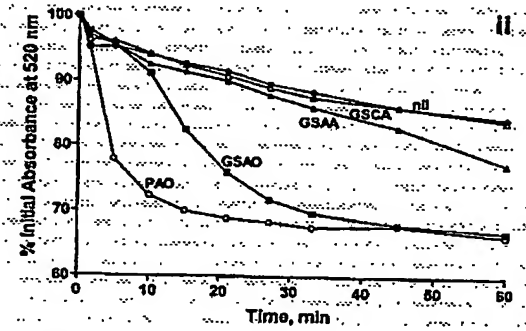
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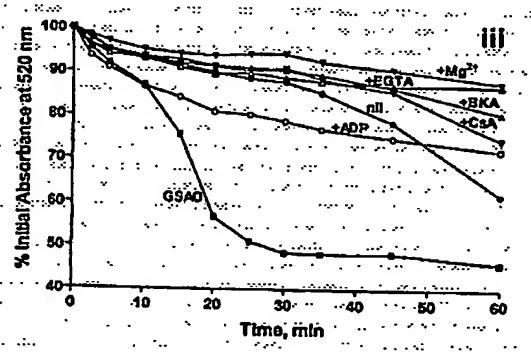
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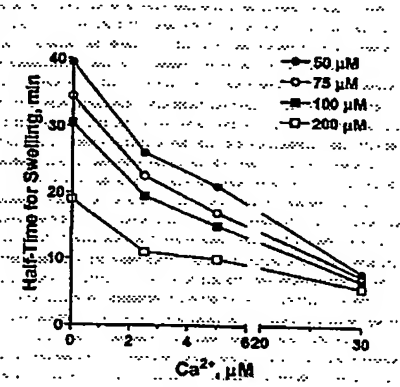
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Half-Time for Swelling, min
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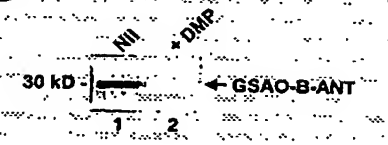
75 μM
100 μM
200 μM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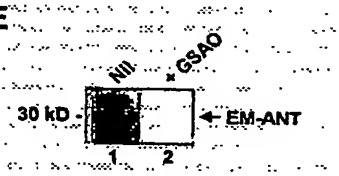
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Figure 1

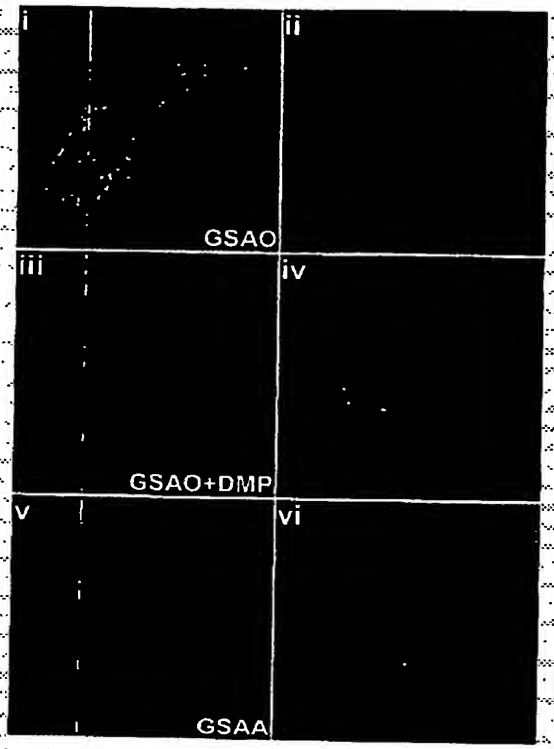
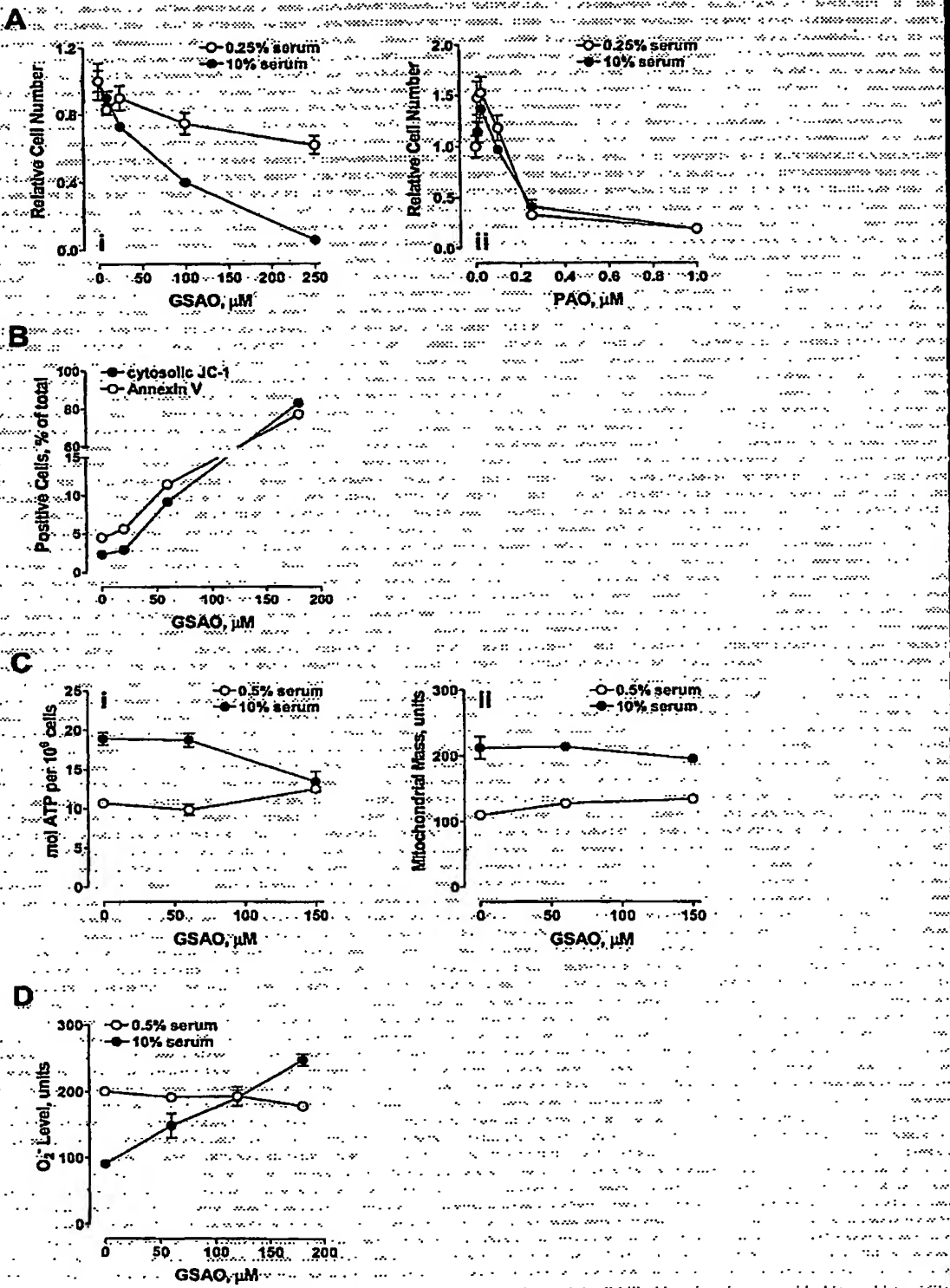


Figure 2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10% serum
Relative Cell Number
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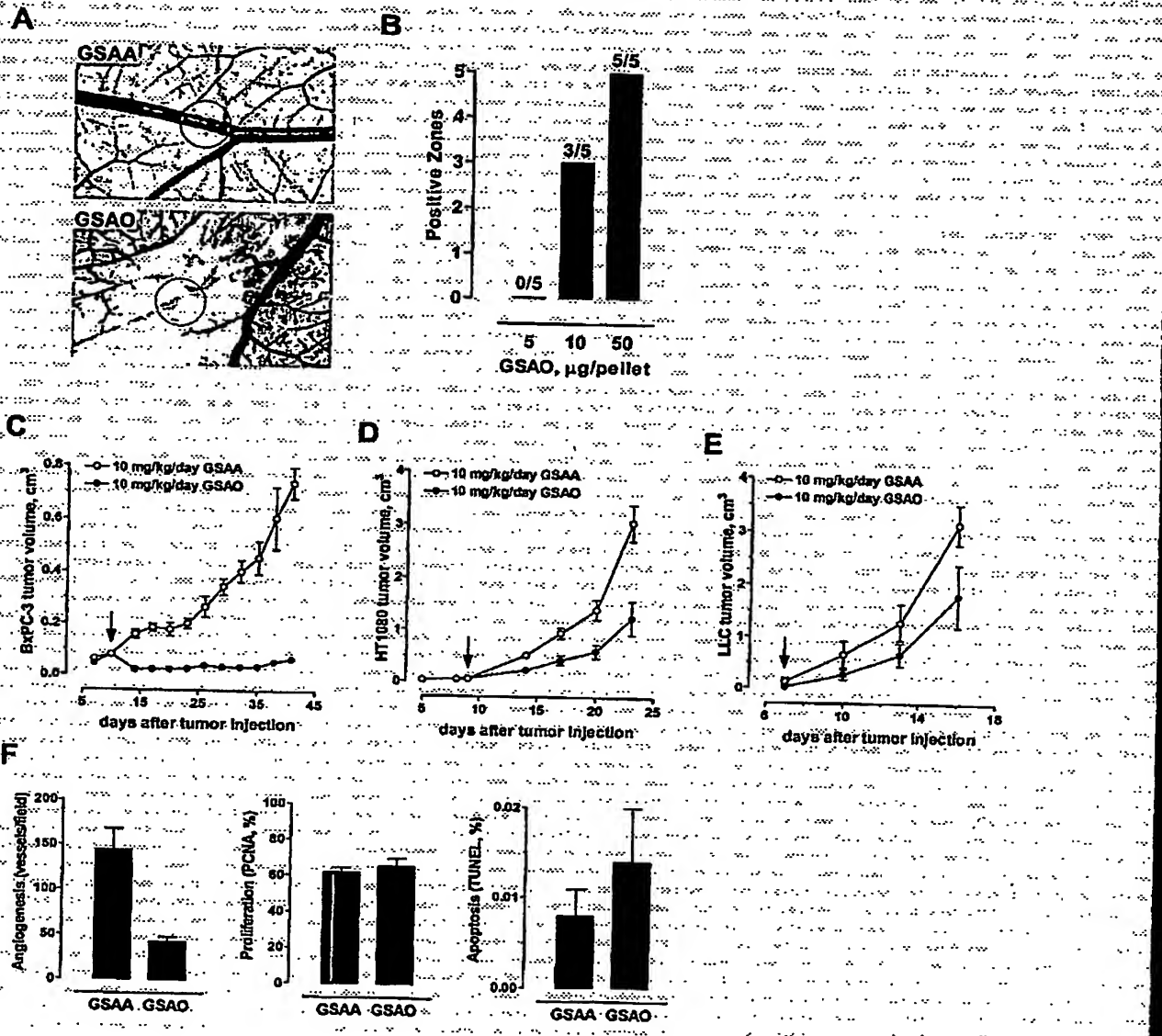
GSAO, μM
ii
PAO, μM
B
cytosolic JC-1
Annexin V
Positive Cells, % of total
GSAO, μM
C
0.5% serum
10% serum
i
mol ATP per 10⁶ cells
GSAO, μM
ii
Mitochondrial Mass, units
GSAO, μM
D
0.5% serum
10% serum
O2- Level, units
GSAO, μM

Figure 3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10 mg/kg/day GSAO
BxPC3 tumor volume, cm³
days after tumor injection
D
HT1080 tumor volume, cm³
days after tumor injection
E
LLC tumor volume, cm³
days after tumor injection
F
Angiogenesis (vessels/field)
Proliferation (PCNA, %)
Apoptosis (TUNEL, %)
GSAA GSAO

Figure 4